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REVIEW ARTICLE

Caspases in retinal ganglion cell death and axon regeneration

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Retinal ganglion cells (RGC) are terminally differentiated CNS neurons that possess limited endogenous regenerative capacity after injury and thus RGC death causes permanent visual loss. RGC die by caspase-dependent mechanisms, including apoptosis, during development, after ocular injury and in progressive degenerative diseases of the eye and optic nerve, such as glaucoma, anterior ischemic optic neuropathy, diabetic retinopathy and multiple sclerosis. Inhibition of caspases through genetic or pharmacological approaches can arrest the apoptotic cascade and protect a proportion of RGC. Novel findings have also highlighted a pyroptotic role of inflammatory caspases in RGC death. In this review, we discuss the molecular signalling mechanisms of apoptotic and inflammatory caspase responses in RGC specifically, their involvement in RGC degeneration and explore their potential as therapeutic targets.

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BULLET POINTS

- Caspase-mediated cell death can occur in normal physiology and pathology.
- Retinal ganglion cells undergo caspase-mediated apoptosis.
- Pyroptosis, a specialised form of inflammatory programmed cell death, mediated by inflammatory caspases, can occur in retinal ganglion cells.
- Inhibition of caspases with pharmacological or genetic inhibitors promotes retinal ganglion cell survival.

INTRODUCTION

Retinal ganglion cells (RGCs) in the ganglion cell layer (GCL) of the inner retina form axons of the optic nerve (ON), which partially decussate at the optic chiasm, project in the optic tract and synapse in the lateral geniculate nucleus (LGN) as well as the superior colliculus, pretectal nucleus and hypothalamus. Optic radiations relay visual information from the LGN to the visual cortex.[1] The neural retina is an outgrowth of the central nervous system (CNS); consequently after injury, there is limited endogenous axon regeneration and lost RGCs are not replaced, leading to irreversible visual loss.

Caspases, a family of cysteine aspartate proteases, have roles in neuronal pruning during development, inducing RGC death (through apoptosis and pyroptosis) after trauma and disease and promoting RGC axon regeneration. Such processes are attenuated by endogenous and pharmacological inhibitors as well as gene knockdown using short interfering RNA (siRNA) to both understand signalling mechanisms and develop therapeutics to prevent RGC death and promote axon regeneration.

Here we review caspases in apoptotic and pyroptotic RGC death, the novel role of caspases in RGC axon regeneration and the neuroprotective success of caspase-targeting interventions.

CASPASES

Caspases are cysteine aspartate proteases that can be divided into two major phylogenic subfamilies, either interleukin (IL)-1β-converting enzyme (inflammatory) or mammalian counterparts of CED-3 (apoptotic) caspases.[2,3] Caspases are the main components of the apoptotic signalling cascade, although they do also have other non-apoptotic roles, including inflammation.[4,5] Caspases are activated by proximity-induced dimerisation, within protein complexes, feedback loops and pro-enzyme cleavage.[6,7]

Apoptotic caspases

Caspases induce apoptosis through initiator and executioner family members: initiator caspases (caspase-2, -8, -9 and -10) activate executioner caspases (caspase-3, -6 and -7) through catalytic cleavage of their activation domain.[5,8] Activated executioner caspases then hydrolyse or cleave proteins leading to cellular apoptosis.[2]

Caspases can be activated through the canonical intrinsic or extrinsic apoptotic pathways (Figure 1). The extrinsic pathway is activated through ligand-activation of tumour necrosis factor (TNF) receptor members[9] including Fas/CD95 receptor, successive recruitment of adaptor proteins, such as Fas-associated protein with death domain (FADD)[9,10] and subsequently pro-caspase-8.[11] Interactions between Fas/CD95, FADD and caspase-8 form the death-induced signalling complex (DISC)[9,12] and initiate caspase-8 activation,[11,12] which sequentially cleaves and activates executioner caspase-3, -6 and -7.[5] Additionally, caspase-8 can cleave the B-cell lymphoma (Bcl)-2 protein family member BH3 interacting domain death agonist (Bid) into truncated Bid (tBid), which stimulates mitochondrial outer membrane permeabilisation (MOMP), releasing apoptogenic factors,[13] including Cytochrome *C*, apoptotic protease activating factor 1 (Apaf-1), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), high-temperature requirement (Htr) A2 (also known as Omi), endonuclease-G and apoptosis-inducing factor.[14,15]

The intrinsic pathway is mitochondria-dependent and activated by intracellular insults, including DNA damage and loss of extracellular membrane integrity, causing MOMP.[13] Mitochondrial-derived Cytochrome *C* complexes with Apaf-1, recruits and activates pro-caspase-9 in a protein complex termed the apoptosome,[16,17] allowing successive activation of

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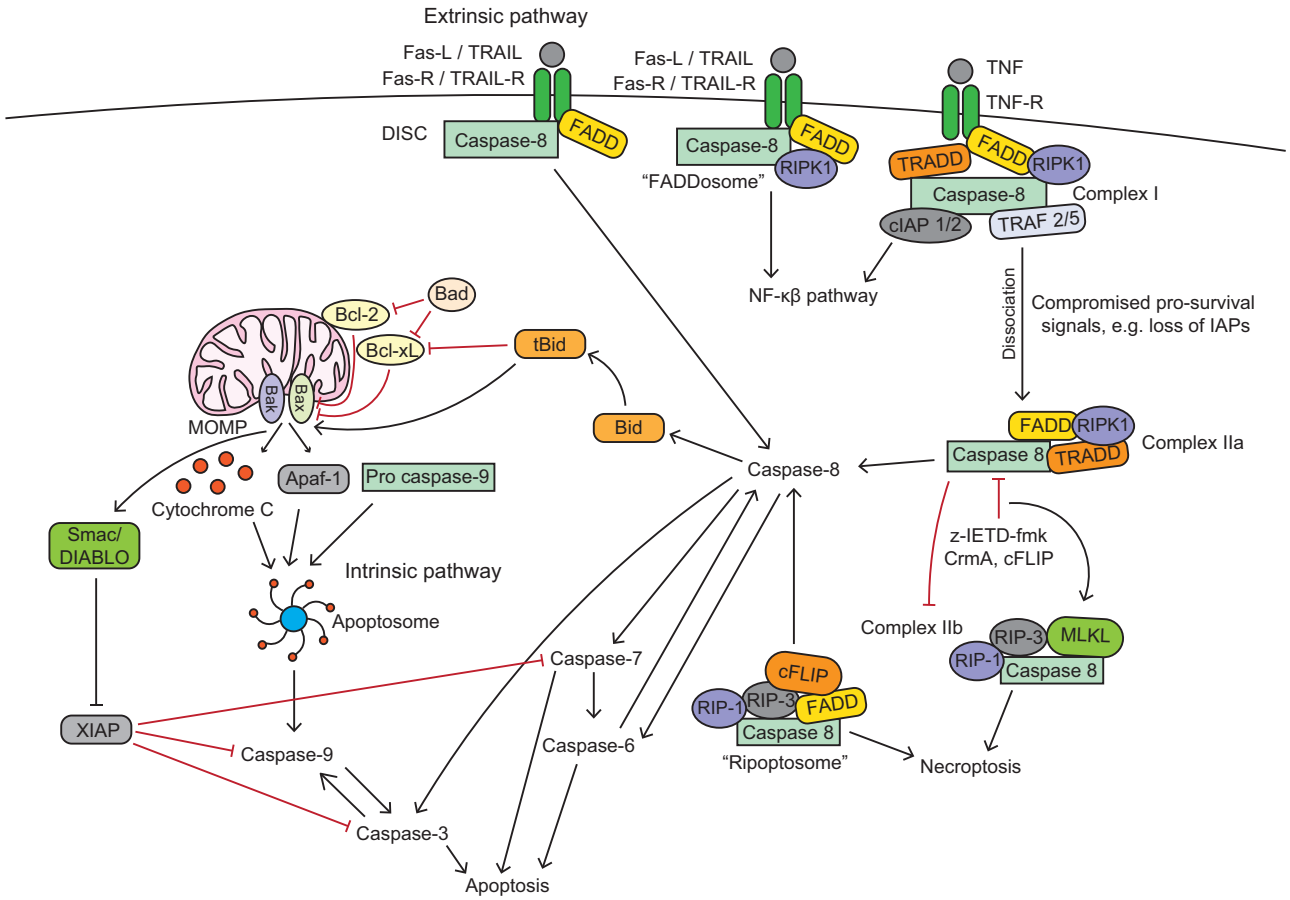

Figure 1. Apoptotic caspases in the canonical intrinsic and extrinsic pathways. Death receptor activation mediates the extrinsic pathway. Fas-R and TRAIL-R recruit FADD[9,10] and pro-caspase-8,[11] forming the DISC,[9,12] leading to proximity-induced caspase-8 activation[11,12] and downstream activation of executioner caspase-3, -6 and -7.[5] Caspase-8 can also activate the intrinsic pathway through truncating BH3-interacting domain death agonist (Bid) into tBid, which then promotes Bak and Bax mitochondrial membrane insertion, increasing MOMP and releasing apoptogenic factors,[13] including Apaf-1, Cytochrome *C* and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO).[14,15] Cytochrome C, Apaf-1 and pro-caspase-9 form the septameric apoptosome complex,[16,17] which activates caspase-9 and successively downstream executioner caspases. Smac/DIABLO indirectly promotes apoptosis by opposing XIAP inhibition of caspase-3, -7 and -9.[22] Caspase-8 can also form complex I at the TNF receptor, which upregulates the NF-κB survival inflammatory pathway; however, if survival signals are compromised (for example, IAPs) then complex I dissociates from the receptor forming complex IIa, which initiates caspase-8-dependent apoptosis.[19] Caspase-8 inhibits complex IIb formation and necroptosis and caspase-8 inhibition (for example, through z-IETD-fmk) induces complex IIb formation, causing necroptosis.[20] The 'ripoptosome' complex forms after cellular IAPs (cIAPs) or XIAP inhibition, causing caspase-8-dependent apoptosis and necroptosis.[23,24]

downstream executioner caspases.[16] TNF cell surface death receptors and different intracellular complexes also mediate cell death (Figure 1). After TNF-R stimulation, receptor interacting protein kinase (RIPK) 1, TNF-R1-associated death domain protein (TRADD), TNF-R associated factor (TRAF 2/5) and cellular inhibitor of apoptosis (cIAP 1/2) are recruited and form membrane-associated complex I.[18] TNF-R primarily drives inflammatory gene transcription through the nuclear factor kappa-light-chain-enhancer of B cells (NF-κB) pathway. Reduced pro-survival signals at the TNF-R (for example, loss of IAPs), dissociates complex I causing RIPK1, TRADD, FADD and caspase-8 to form complex IIa, which initiates apoptosis by caspase-8 auto-activation.[19] Caspase-8 also represses necroptosis (regulated necrosis; mediated by RIPK1 and RIPK3), thus, if caspase-8 is compromised or inhibited, for example, through mammalian inhibitors (CrmA and cFLIPs), pharmacological inhibition (e.g., z-VAD-fmk or z-IETD-fmk) or gene loss, then necroptosis ensues.[20] Necroptosis activation requires RIPK1, RIPK3 and mixed lineage kinase domain-like protein (MLKL), which form complex IIb.[21] X-linked IAP (XIAP) directly inhibits caspase-3, -7 and -9[22] and inhibition of cIAPs and XIAP causes complex II (the 'ripoptosome'; (RIPK1-RIPK3-FADD-caspase-8-cFLIP),[23,24] which drives caspase-8-mediated apoptosis or caspase-independent necroptosis without the need for receptor ligation.

Caspase-8 also acts as a non-enzymatic scaffold in the assembly of a pro-inflammatory 'FADDosome' (caspase-8-FADD-RIPK1) complex, inducing NF-κB-dependent inflammation.[25]

Uniquely, caspase-2 can act as both an initiator and an executioner caspase, depending on the apoptotic stimuli and does not fit into either the classically described intrinsic or extrinsic apoptotic pathways (Figure 2)[26,27]; its structure resembles that of an initiator caspase due to its caspase recruitment domain but can act as an executioner caspase in response to multiple triggers, including DNA damage, heat shock, endoplasmic reticulum and oxidative stress.[28–32] DNA damage induces PIDDosome formation: a protein complex that consists of adaptor protein RIP-associated ICH-1 homologous protein with a death domain (RAIDD)[33] and p53-induced protein with a death domain (PIDD),[30,34,35] which recruit and activate pro-caspase-2. Caspase-2 can also be activated at the DISC. Caspase-2 can also mediate apoptosis directly from the mitochondrial compartment.[36]

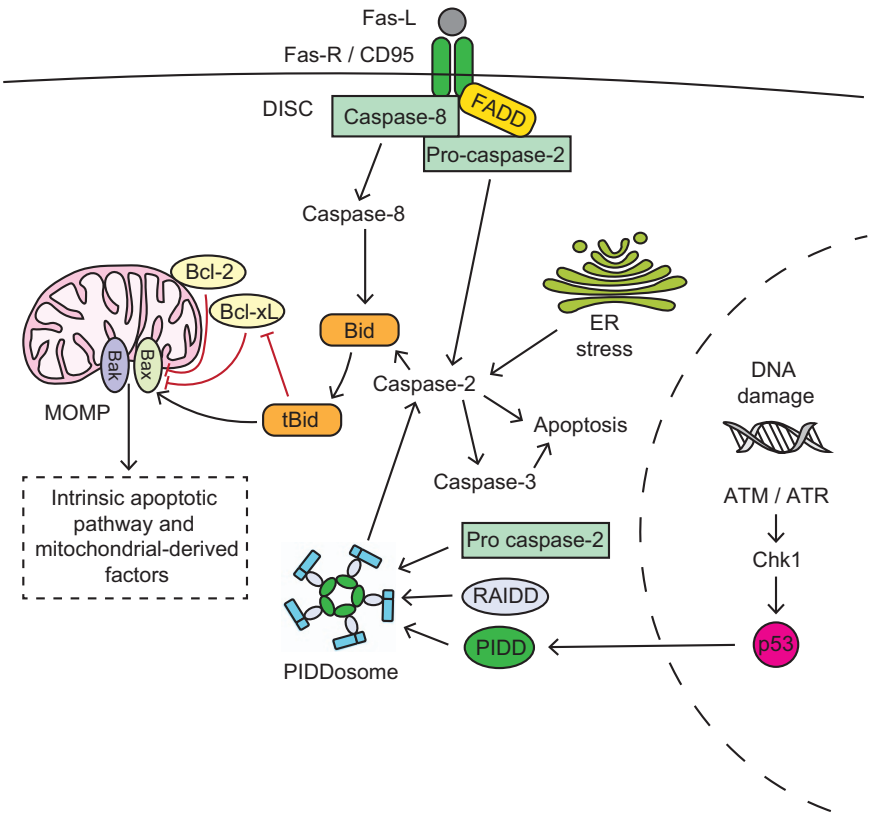

Figure 2. Activation mechanisms of caspase-2. Caspase-2 is activated through DNA damage, upregulation of p53 and formation of the PIDDosome protein complex, which includes p53-induced protein with death domain (PIDD), RIP-associated ICH-1 homologous protein with death domain (RAIDD) and pro-caspase-2.[30,33–35] Caspase-2 is also activated by endoplasmic reticulum (ER) stress and at the Fas-R within the DISC, alongside Fas-associated protein with death domain (FADD) and caspase-8.[28–32] Active caspase-2 cleaves and activates caspase-3, cleaves BH3 interacting domain death agonist (Bid; which initiates MOMP and the intrinsic apoptotic pathway) or initiates apoptosis directly.

Inflammatory caspases

Inflammatory caspases (-1 or -11 in mice and -1, -4 and -5 in humans) can be activated in the inflammasome protein signalling complex (Figure 3).[4,37,38] Inflammasomes are large multimeric protein complexes that sense pathogen- and host-derived danger signals and typically comprise of a Nod-like receptor (NLR), adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1.[37–39] The main functions of the inflammasome are to activate caspase-1 to cleave precursor cytokines IL-1β and IL-18 into their mature active forms and induce pyroptosis (a lytic form of cell death). Active caspase-1 also cleaves gasdermin-D into its cytotoxic N-terminal fragment, which forms a plasma membrane pore, releasing pro-inflammatory cytokines.[40–42] Inflammasome activation is a two-step process: initial inflammasome priming is required for transcriptional upregulation of machinery including Nod-like-receptor pyrin domain containing 3 (NLRP3) and pro-IL-1β,[37,38] followed by the trigger, such as a pathogen-associated molecular pattern (PAMP) or a damage-associated molecular pattern (DAMP), which induces inflammasome assembly and activation.

The canonical NLRP3 inflammasome can be activated by PAMPs (for example, *Staphylococcus aureus*) and host-derived DAMPs (e.g., ATP, phagolysomal rupture, cathepsins release, ion flux, calcium influx, mitochondrial reactive oxygen species and oxidised mitochondrial DNA).[38,43] Potassium efflux has been proposed as a universal trigger for NLRP3 activation,[44] including P2X7 receptor-mediated potassium pore opening, pannexin-1 and pore-forming toxins.[44] However, potassium efflux is not a common mechanism for all activation pathways.[45,46]

Caspase-11, -4 and -5 can be activated by bacterial lipopolysaccharide-induced oligomerisation,[40] cleaving gasdermin-D and indirectly activating the NLRP3 inflammasome via pannexin-1 and potassium efflux.[47] NLRP3 inflammasome can also be activated by caspase-8 – which also directly cleaves IL-1β.[48,49] MLKL translocates to the cell membrane and disrupts it, triggering potassium efflux and assembly of the NLRP3 inflammasome.[50] MLKL activation also provides a mechanism for processing and release of IL-1β independently of gasdermin-D.[50]

ANTICASPASE TREATMENTS: PHARMACOLOGICAL, GENE KNOCKDOWN AND SIRNA TECHNIQUES

A number of specific and broad-spectrum caspase inhibitors are based upon the amino-acid sequence of caspase substrate cleavage sites, acting as pseudoenzymes for active caspases and therefore competitive inhibitors. Broad-spectrum inhibitors include Boc-D-fmk, Q-VD-Oph (inhibits caspase-1, -2, -3, -6, -8 and -9), z-VAD-fmk (inhibits all caspases but caspase-2 very weakly).[51–54] Specific caspase substrate cleavage sites include WEHD (caspase-1), YVAD (caspase-1), VDVAD (caspase-2), DEVD (caspase-3), LEVD (caspase-4), VEID (caspase-6), LETD (caspase-6), IETD (caspase-8 and -10) and LEHD (caspase-9)[53,55,56 2,3]. Caspase peptide inhibitors are linked to chemical groups that improve permeability, efficacy and stability of the compound. Peptides linked to aldehydes (or nitriles or ketones) are reversible inhibitors (e.g., Ac-DEVD-CHO) and bind to the catalytic site but do not irreversibly chemically alter the enzyme, whereas peptides linked to halmethylketones (chloro or fluoro) (e.g., z-VAD-fmk) bind irreversibly. The chemical group -fmk is non-specific.[56,57]

Cross-reactivity with 'off-target' caspases limits interpretation of many studies using these inhibitors. The sequence DEVD (caspase-3) also binds to caspase-6, -7, -8 -9 and -10, similarly

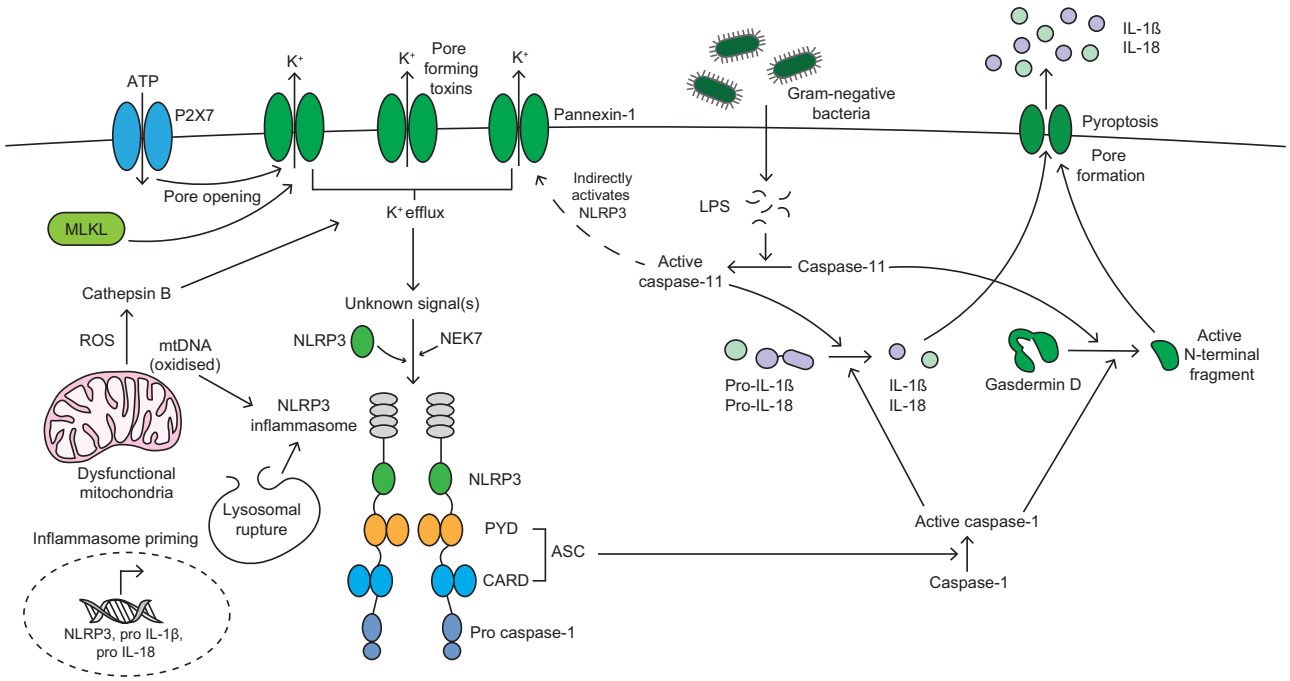

Figure 3. Inflammatory caspase-1 is activated within the inflammasome protein complex;[4,37,38] which typically consists of a Nod-like receptor (NLR; such as Nod-like-receptor pyrin domain containing 3 (NLRP3)), adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1.[37–39] Initial inflammasome priming is required for transcriptional upregulation of inflammasome machinery, such as NLRP3, pro-IL-1β and pro-IL-18.[37,38] A second signal then induces inflammasome assembly and activation. The NLRP3 inflammasome is activated by lysosomal rupture, reactive oxygen species (ROS), oxidised mitochondrial DNA (mtDNA) and cathepsin B.[38,43] Potassium (K^+) efflux is a common NLRP3-activation mechanism, induced by P2X7-mediated pore opening, pore-forming toxins, pannexin-1 or MLKL-mediated pore opening.[44] The NLRP3 inflammasome activates caspase-1, which cleaves precursor cytokines IL-1β and IL-18 into their active forms and gasdermin-D into its N-terminal fragment. The N-terminal fragment of gasdermin-D forms a plasma membrane pore facilitating pro-inflammatory cytokines release and inducing pyroptosis.[40–42] Gram-negative bacterial lipopolysaccharide (LPS) can activate caspase-11,[40] which also cleaves gasdermin-D cleavage and indirectly activates the NLRP3 inflammasome via pannexin-1.[47]

VDVAD (caspase-2) binds caspase-3 and -7 and LETD (caspase-6) binds caspase-3, -8 and -9.[55,58,59] VEID has a stronger efficacy for caspase-3 than its target caspase-6, IETD has a stronger efficacy for caspase-3 and -6 than its target caspases -8 and -10 and LEHD has a stronger efficacy for caspase-8 and -10 than their intended substrate IETD, and LEHD also binds caspase-3 and -6.[55,58,59] In addition, z-VAD-fmk also binds other cysteine proteases, such as calpains and cathepsins.[51]

Caspase activity can also be modulated by siRNA-mediated gene knockdown, dominant-negative proteins and conditional and global gene knockout. RNA interference technology may cause alternative signalling induced by short RNA species and off-target effects, thus appropriate controls are still critical.[60]

CASPASES AND RGC DEATH

Caspase-dependent RGC death occurs after eye and brain injuries, in retinal and optic nerve degenerative disorders[61,62] and during development.[63,64] Common mechanisms of degeneration between different conditions could lead to broadly translatable therapeutics. Caspase involvement in RGC death in animal models, primary cell culture and human postmortem specimens are highlighted in this section. Relative efficacy of neuroprotection is shown for direct caspase inhibitors in Table 1 and upstream indirect inhibitors in Table 2.

Endogenous caspase activity and inhibition in RGC

Development. Caspase-dependent apoptosis is important in pruning neuronal, including RGC, numbers after normal developmental overproduction,[63,65] causing an ~50% reduction in RGC numbers shortly after cell birth, which can be prevented by broad-spectrum caspase inhibitor, Boc-D-fmk.[66,67] Caspase-3 is pivotal in neuronal developmental apoptosis, with active caspase-3 co-localising to terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive RGC in 2–6-day chick embryos,[67] and caspase-3 inhibition, using z-DEVD-fmk, reducing TUNEL-positive cells by ~50% and increasing RGC numbers, axons and GCL thickness.[67] Moreover, BARHL2, a member of the *Barh* gene family, which suppresses caspase-3 activation, is essential for developmental preservation of normal complement of RGC subtypes.[68]

Supporting this, caspase-3 knockout mice express a brain-specific phenotype with excessive neuronal numbers and cellular disorganisation, dying at 1–3 weeks of age.[3,69] Similarly, caspase-9 knockout results in a selective CNS phenotype, characterised by severe brain malformations and high perinatal lethality without gross abnormality of other body parts.[70,71] Caspase-2 (NEDD2) gene expression is elevated during neurogenesis and down-regulated in the mature brain and retina.[72,73] However, caspase-2 knockout mice develop normally and lack overt phenotypic abnormalities, with minimal CNS or retinal defects. The role of caspase-2 in RGC neurogenesis is therefore unclear. In more mature mouse retinae, there are no alterations in caspase-3, -6, -7, -8 or -9 expression between 6 and 24 weeks.[74] However, there was a reduction in cIAP-1 suggesting a possible role for caspases at this stage.[74]

Induced caspase activity and anti-caspase treatment in RGC

Optic neuritis. Multiple sclerosis (MS) is an autoimmune, demyelinating CNS disease and a major cause of non-traumatic disability in young adults. Optic neuritis involves ON inflammation and

Table 1. Treatments directly targeting caspases in RGC degenerative disease

Caspase	*Model*	*Inhibitor*	*Time at the end of the study (days)*	*Percentage surviving RGC (% untreated)*	*Percentage surviving RGC (% treated)*	*References*
Broad spectrum	ONT	z-VAD	14	16.8[a]	34.5[a]	61
	75 min raised IOP	Q-VD-OPH	7–21	39–64	63–71	62
Caspase-1	ONC	NLRP3 −/−	3–28	78–13	89–25	108
	NMDA-RGC explants	YVAD-fmk	2	18	12	135
Caspase-2	ONC	z-VDAD-fmk	15	12.3	60	95,96
	ONC	siCASP2	21–84	10–7	95–96	91,116
	Optic neuritis	siCASP2	21	65.5	79.3	81
Caspase-3	ONT	z-DEVD-cmk	7–28	10–34.3	24.3–47.4	61,100,101
	NMDA-RGC explants	DEVD-fmk	2	18	26	135
Caspase-3 and -6	NMDA-RGC explants	DQMD-fmk	2	18	41.6	135
Caspase-6	ONT	SIMA 13a	13	16.8[a]	37[a]	61
	ONC	CASP6 DN	21	14.2	39.4	96
	ONT	z-VEID	14	16.8[a]	48.2[a]	61
	NMDA-RGC explants	VEID-fmk	2	18	41.6	135
	30 min artery ligation	z-VEID-fmk	14	33.9	46.2	161
	30 min artery ligation	siCASP6	14	30[a]	48[a]	161
Caspase-7	ONT	CASP7 −/−	28	38	76	106
Caspase-8	ONT	z-IETD (+/−) -fmk	14	16.8[a]	31.5–60.7[a]	61,97
	ONT	IETD-CHO	14	NA	33.1	97
	NMDA-RGC explants	IETD-fmk	2	18	27	135
	30 min artery ligation	z-IETD-fmk	14	33.9	42.2	161
	30 min artery ligation	siCASP8	14	30.0[a]	48.4[a]	161
Caspase-8 and -9	ONT	z-IETD-fmk and z-LEHD-fmk	14	NA	38.7	97
Caspase-9	ONT	z-LEHD- (+/−) fmk	14	16.8[a]	29.1–34.9[a]	61,97
	NMDA-RGC explants	LEHD-fmk	2	18	39	135

Specific pharmacological inhibitors, gene knockdown (i.e., siRNA) or gene knockout (−/−) treatment are displayed with the percentage of surviving RGC in untreated and treated retinae. [a]For calculations, values for uninjured Fluoro-Gold and RBPMS RGC counts not stated in Shabanzadeh *et al*.[161] and values for identical animals (Sprague Dawley female adult rats) with Fluoro-Gold and RGC counts per mm^2 were used from Weishaupt *et al*.[97]

demyelination and is a common presenting feature of MS[75] associated with visual loss. The extent of visual recovery after acute optic neuritis is influenced by demyelination, axonal loss and RGC death.[76] The experimental autoimmune encephalomyelitis (EAE) model is the most common MS animal model induced by myelin oligodendrocyte glycoprotein (MOG) peptide administration causing autoimmunity, inflammation and neurodegeneration.[77,78] In the EAE rat model cleaved caspase-3 immunolocalised to Fluoro-Gold-labelled RGC suggesting that RGC die by apoptosis,[77] though in the EAE mouse model only full-length caspase-3 immunostaining is present in the GCL.[78] RGC NADH dehydrogenase (mitochondrial electron transport chain) overexpression suppresses RGC death, rescuing 88% of RGC and reducing cleaved caspase-3 immunostaining in Thy1-labelled RGC.[79] Treatment with erythropoietin (EPO) reduces RGC death and active caspase-3 levels, supporting a critical role for caspase-3.[80] Various regulators upstream of caspase-3 are also neuroprotective (Table 2).

In a refined mouse model of MS, the MOGTCR × Thy1CFP mouse, which develops optic neuritis only, either spontaneously or following induction with Bordetella pertussis toxin,[81] RGC express active caspase-2 and intravitreal injection of a modified siRNA against caspase-2 (siCASP2) protects ~80% of RGC against apoptosis and axonal degeneration,[81] suggesting a critical role for caspase-2 in RGC apoptosis after optic neuritis.

Traumatic optic neuropathy. Traumatic optic neuropathy (TON) is a major cause of visual loss after brain and eye injury. TON can be either direct – when the ON is crushed or severed – or more commonly indirect, when brain or ocular injury causes secondary RGC death or ON injury. Spontaneous recovery occurs in a minority of patients.[82] However, the most common outcome is permanent blindness, and at present, there is no treatment that improves outcome.[83,84] Direct TON can be caused by penetrating injury, such as craniofacial fractures, or direct compression from orbital haemorrhage.[85] ON transection (ONT) and ON crush (ONC) in animal models can be used to study degenerative mechanisms and evaluate neuroprotective and regenerative therapies.[86,87]

RGC death after ON injury is progressive and the severity is dependent upon type of lesion and distance from the eye.[88,89] After direct TON, RGC begin to degenerate 5 days after axotomy,[90] and 90% die between 7 and 14 days[86,89,91,92] through caspase-dependent apoptosis.[93,94] Cleaved caspase-2,[91,95,96] -8,[61,97] -9,[90,98,99] -3,[90,100–105] -6[61] and -7,[102,106] as well as inflammatory caspases -11[107] and -1,[108] have all been detected in RGC after crush or axotomy, highlighting the crucial role played by caspases in axotomy-induced RGC death.

Caspase-3 is activated after RGC axotomy,[90,100–105] and z-DEVD-fmk inhibition reduces RGC death.[99,101–103,109,110] However, z-DEVD-fmk also inhibits caspase-6, -7, -8 -9 and -10[55,59] and neither delayed nor multiple treatments of z-DEVD-fmk improved the RGC survival.[101] Caspase-3 is also indirectly reduced in RGC-neuroprotective therapies, such as either Rho-associated protein kinase (ROCK) inhibition[111,112] or treatment with the broad-spectrum histone deacetylase inhibitor, valproic acid.[113,114] Moreover, a rabbit fluid percussion injury model of indirect TON increases cleaved caspase-3 in retinal lysate, where full-length caspase-3 is localised to RGC and pharmacological inhibition with z-DEVD-fmk is RGC neuroprotective.[115]

Caspase-7 gene knockout also protects a limited proportion of RGC after axotomy[106] and pharmacological inhibition of caspase-6 and -8, using z-VEID-fmk and z-IETD-fmk or a dominant-negative against caspase-6 (CASP6 DN) provides some RGC neuroprotection and promotes regeneration.[61] Although caspase-6 is localised to RGC and some microglia, regeneration is an indirect effect of ciliary neurotrophic factor (CNTF) production by retinal glia.[96]

Table 2. Treatments that affect targets upstream of caspases and prevent RGC death

Disease	*Injury*	*Treatment*	*Effect on caspase by treatment*	*End of the study (days)*	*Effect on RGC*	*References*
Direct ON injury	ONC	ROCK inhibition	Reduced cleaved caspase-3 immunostaining in GCL and primary RGC culture lysate	14	ROCK shRNA increases RGC survival to 143% of EGFP shRNA control	111,112
	ONC	Calcineurin inhibition	Reduced cleaved caspase-9 protein	—	ND	98
	ONC	Deletion of CHOP	Reduced full-length caspase-3 immunostaining	14	CHOP KO mice had 52% surviving RGC compared with 24% in sham	198
	ONT	Kv1.3 siRNA	Reduced caspase-3 and -9 mRNA expression	14	KV 1.3-1169 siRNA increases RGC survival 3.5-fold compared with control	199
	ONC	Valproic acid (VPA)	Reduced cleaved caspase-3 RGC immunostaining	14	VPA treatment has 44% surviving RGC compared with 27% in vehicle	113,114
Glaucoma	Hypertonic saline injections into limbal vein	Morphine	Reduced cleaved caspase-3 and -8 protein	56	Morphine treatment has 65.9% surviving RGC compared with 17.3% in control	166
	Laser photocoagulation	Cobra venom factor (CVF; complement depletion)	Reduced cleaved caspase-8 and -9 protein	42	CVF treatment has 41.5% surviving RGC compared with 28.4% in control	165
	Suture pulley compression	C-Jun N-terminal kinase (C-JNK) inhibitor	Reduced cleaved caspase-3 immunostaining	0.5	C-JNK inhibition has 23.6% of RGC as TUNEL positive compared with 52.4% in vehicle control and 1.49% in uninjured	167
	Saline injection into anterior chamber	Cyclosporine A (CSA; inhibits cyclophilin D and MPTP)	Reduced cleaved caspase-3 protein, immunolocalised to RGC	14	CSA treatment has 93% surviving RGC compared with 77% in ischaemic	200
	Translimbal photocoagulation laser model	Minocycline, tetracycline antibiotic	Reduced caspase-1 and -4 but not caspase-8 and -12 gene expression	8	ND	170,201
Glutamate excitotoxicity	Glutamate – primary rat RGC culture	Pilocarpine (M1 muscarinic receptor agonist)	Reduced caspase-3 gene expression and full-length protein	1	Cell viability is 42% after 1 mM of glutamate, increases by 32% with pilocarpine treatment	153,202
	NMDA administration	Thioredoxin (TRX)	Reduced cleaved caspase-3 and -9 protein	7	TRX treatment has 56.6% surviving RGC compared with 13.4% in control	203
Ischaemic injury	Ischaemic reperfusion injury	Brain-derived neurotrophic factor (BDNF)	Reduced full-length caspase-2 GCL immunostaining	7	BDNF treatment has 69.6% surviving RGC compared with 44.1% in sham	153,154
	Ischaemic reperfusion injury	VPA	Reduced cleaved caspase-12 protein	7	VPA treatment has 83.5% surviving GCL cells compared with 57.5% in sham	157
Branch retinal vein occlusion (BRVO)	Laser photocoagulation	Minocycline, tetracycline antibiotic	Reduced cleaved caspase-3 immunostaining in GCL	7	*In vivo* OCT imaging shows increased RNFL +GCL thickness 3 days after minocycline. Minocycline has 55.2% RGC compared with 46.8% in saline control	204
Diabetic retinopathy	STZ	Somatostatin (SST)	Reduced cleaved caspase-8 and -3 protein	14	Reduced TUNEL cells in GCL, 36.8% in STZ compared with 13.7% in treated	195
	STZ	Edaravone (free radical scavenger)	Reduced cleaved caspase-3 protein	28	Reduced TUNEL cells in GCL, 42% in vehicle compared with 9.5% in treated	205
	High glucose primary RGC culture	Erythropoietin (EPO; antioxidant)	Reduced full-length caspase-3 and -9 protein	—	Reduced apoptotic Hoechst 33358-stained cells, 49.1% in high glucose compared with 25.7% in EPO treated	206
	High glucose primary RGC culture	L-Carnitine (endogenous mitochondrial membrane compound)	Reduced full-length caspase-3 and -9 protein	—	Reduced apoptotic Hoechst 33358 stained cells, 49.1% in high glucose compared with 15.7% in L-Carntine treated	207
Optic neuritis	EAE model	EPO	Reduced cleaved caspase-3 immunostaining	8	EPO treatment has 55% RGC surviving compared with 30% in vehicle control	80
PBI	Blast wave	Compound 49b (beta-adrenergic receptor agonist)	Reduced cleaved caspase-3	3	ND	125

Abbreviations: CHOP, CCAAT/enhancer binding homologous protein; EAE, experimental autoimmune encephalomyelitis; MPTP, mitochondrial permeability transition pore; NMDA, *N*-methyl-d-aspartate; PBI, primary blast injury; ROCK, Rho-associated protein kinase; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

In addition, combined caspase-8 and -9 inhibition provides additive survival benefits compared with single inhibition,[90,97,102] which may suggest either that both intrinsic and extrinsic apoptotic pathways are activated following direct optic nerve injury or that there are increased off-target effects. Inhibition of caspase-8 can also promote caspase-independent RGC death, such as necroptosis.[20]

Recent studies have indicated a pivotal role of caspase-2 in apoptotic RGC injury.[91,95,96,116,117] After ON axotomy and crush, active caspase-2 is exclusively localised to RGC, and its inhibition using siRNA provides significant neuroprotection.[91,95,96] For example, intravitreal administration of either siCASP2[91] or the pharmacological inhibitor z-VDVAD-fmk[95] protect 98% and 60% of RGC, respectively, for up to 30 days and >95% of RGC are protected from death for 12 weeks if siCASP2 is injected every 8 days.[116] Pharmacological inhibition with z-VDVAD-fmk also inhibits caspase-3 and -7,[59] though activation of these caspases was not affected. The siCASP2 is being developed by Quark Pharmaceuticals Inc. and is currently in Phase III clinical trials for ischaemic optic neuropathy and glaucoma.[116]

NLRP3-induced neuroinflammation promotes RGC death after partial ONC.[108] NLRP3 expression is upregulated in retinal microglia and NLRP3 inflammasome activation upregulates retinal cleaved caspase-1 and IL-1β, which is prevented in NLRP3 knockout mice, in which RGC are protected against axotomy-induced RGC death.[108] The P2X7 ionotropic ATP-gated receptors are implicated in RGC degeneration; P2X7-mediated potassium efflux induces NLRP3 inflammasome formation and caspase-1 activation.[44] P2X7 receptor-deficient mice displayed delayed RGC loss and reduced phagocytic microglia at early time points after RGC axotomy.[118] Intravitreal administration of a selective PX27 receptor antagonist A438079 delayed RGC death, suggesting P2X7 receptor antagonism as a potential therapeutic strategy.[118] Caspase-11 expression is also upregulated in RGC after ONC and ONT.[107]

Primary ocular blast injury. Although direct ON injury results in rapid RGC degeneration, indirect blast-induced TON is delayed and progressive. After explosive blast, the sonic blast-wave causes primary blast injury (PBI), which can cause indirect TON.[119,120] Secondary blast injury causes direct and indirect TON, when explosively propelled fragments impact the eye, head and ON. Blast injury represents a significant threat to military personnel in modern warfare causing visual loss.[121,122] Multiple studies have demonstrated increased cleaved caspase-3 in the GCL and ON between 3 and 72 h after whole animal[123,124] and direct local ocular blast exposures.[125] Moreover, caspase-3 activation displays a cumulative effect after multiple exposures,[124] which is comparable to repeated exposure in combat, potentially leading to worse structural and functional visual outcomes.[126] Additionally, an alternative model using trinitrotoluene (TNT) explosives detected active caspase-3 exclusively in photoreceptors and not RGC.[127] Other apoptotic markers, such as Bax, Bcl-xL and Cytochrome *C* are also elevated in the retina up to 24 h after blast injury.[125] DBA/2J mice lack ocular regulatory mechanism of immune privilege in the anterior chamber,[128] and are thus used as a closed globe injury model to approximate features of open globe injury, without complications of infection.[129] In this model, full-length inflammatory caspase-1 is immunolocalised to the inner nuclear layer (INL) and GCL in control retinae, but immunostaining declines after blast injury,[129] suggesting caspase-1 cleavage. However, necroptotic markers RIPK1 and RIPK3 have increased retinal expression, with RIPK1 localised to outer nuclear layer (ONL), INL and Müller glia and RIPK3 in the ONL, INL and GCL 3 and 28 days post-ocular PBI.[130] These findings suggest potential activation of necroptotic or pyroptotic death pathways.

Although caspase activation immediately follows blast injury, RGC death does not occur until later time points,[130] with retinal nerve fibre layer (RNFL) thickness unchanged for 3 months postblast.[131,132] Axonal degeneration at 28 days after ON demyelination[130] suggests that, as in direct TON, ON degeneration may precede RGC death.[133] Research into blast-induced RGC degeneration is in its infancy. However, roles for apoptotic and potentially inflammatory caspases in RGC death are apparent.

Excitotoxicity-induced RGC death. Excitatory neurotransmitter glutamate is linked to retinal degeneration, for example, in glaucoma, through overactivation of *N*-methyl-D-aspartate (NMDA) receptors, calcium overload and subsequent mitochondrial dysfunction. Excitotoxicity-induced RGC death is caspase dependent; broad-spectrum caspase inhibition preserves GCL cells.[134] Intravitreal caspase-3, -6, -8 and -9 inhibitors, DEVD-fmk, VEID-fmk, IETD-fmk and LEHD-fmk respectively, significantly protect RGC, but caspase-1 and -4 inhibition, using YVAD-fmk, does not,[135] suggesting that excitotoxicity-induced RGC death is apoptotic but not pyroptotic. The greatest RGC neuroprotection is provided by DEVD-fmk, which inhibits caspase-3 and also -2, -6, -7, -8, -9 and -10. The latter, LEHD-fmk (intended for caspase-9), is most specific for caspase-3 and -8 and also inhibits -6 and -10.[58,59,135]

The IQACRG amino-acid sequence is conserved in the active site of caspase-1, -2, -3, -6 and -7 and the synthetic peptide, with amino-acid sequence IQACRG, acts as an enzymatically inactive caspase mimetic, thus binds to caspase substrates as a pseudo-enzyme and protects them from proteolysis by caspases. Treatment with IQACRG caspase mimetic protects RGC from excitoxicity-induced death both *in vivo* and in primary culture.[136]

Light-induced retinopathy. Light exposure can cause light-induced retinal damage (LIRD) and blindness,[137,138] and a light-toxicity animal model induces photoreceptor and caspase-dependent RGC apoptosis.[139] Cleaved caspase-3 is elevated in RGC 6 h after toxic light exposure and reaches a peak after 3 days,[140–142] co-localising with increased staining for Ras homologue enriched in the brain (RHEB), cyclic AMP response element modulator-1 (CREM-1), transcription initiator factor IIB (TFIIB), pyruvate kinase isozyme type M2 (PKM2), SYF2 pre-mRNA splicing factor (SYF2) and RNA-binding motif protein, X-linked (RBMX), which are all involved in cell death pathways.[140–145] Nuclear factor of activated T cells, cytoplasmic 4 (NFATc4) (a component of T-cell activation and a regulator of the immune response) are also co-localised with cleaved caspase-3, caspase-8 and Fas-L in RGC, suggesting that NFATc4 may upregulate Fas-L and participate in RGC apoptosis.[146] Intravitreal mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) inhibitor reduces PKM2 and active caspase-3 protein expression, suggesting that light-induced RGC apoptosis is in part dependent on MAPK/ERK pathway.[141] Together, these studies show that RGC apoptosis is correlated with caspase-3 cleavage but not that RGC death in LIRD is caspase-3 dependent.

Ischaemic RGC death. Retinal ischaemia is a common cause of visual impairment and sight loss[147] and can be experimentally induced by clamping or ligation of the ophthalmic artery, raising intraocular pressure (IOP) or bilateral common carotid artery occlusion.[148–151] The degree of RGC loss after ischaemic injury is dependent upon the length of ischaemic interval and is progressive. For example, after 45 min of ligation, ischaemia induces ~50% of RGC to degenerate over a 2-week period, whereas 120 min induces death of 99% over 3 months.[151]

Ischaemic RGC degeneration is caspase dependent, evidenced by neuroprotection with broad-spectrum caspase inhibitors (Q-VD-OPH and Boc-aspartyl-fmk).[62] In Thy1-positive RGC, full-length caspase-2 expression is increased 1,[152] 6,[153,154] 24[152,154] and 72 h[152] after ischaemia and antisense oligonucleotide inhibitor of caspase-2 (antisense Nedd-2 oligonucleotide 5′-QGCTCG GCGCCGCCATTTCCAGL-3′) protected inner retinal thickness at 7

days.[152] Brain-derived neurotrophic factor (BDNF) is also RGC neuroprotective and reduced caspase-2 expression.[153] Full-length caspase-3 immunolocalised to the GCL 4 h after injury[155] and preinjury intravitreal siRNA caspase-3 injection was RGC neuroprotective,[156] though other studies have found full-length caspase-3 to be exclusively in the INL and ONL.[152] Valproic acid, a broad-spectrum histone deacetylase inhibitor, protects RGC after ischaemic reperfusion (I/R) injury caused by raised IOP,[113,114,157] reducing cleaved caspase-3 and -12 expression.[114,157]

Pannexin-1 is a mammalian cell membrane channel-forming protein that acts as a diffusional pathway for ions and small molecules. Pannexin-1 facilitates neurotoxicity in the ischaemic brain and retinal pannexin-1 gene knockout suppresses inflammasome-mediated caspase-1 activation and IL-1β production 3 h after ischaemic injury and reduces RGC degeneration at 14 days.[158] Administration of YVAD-fmk (caspase-1, -4 and -5) protects inner retinal morphology in some, but not all, studies,[152,154,155] leaving the role of caspase-1 in question. P2X receptor stimulation induces ATP influx, potassium ion efflux and downstream NLRP3 inflammasome and caspase-1 activation.[37,38] During stimulated ischaemia (oxygen/glucose deprivation) of human organotypic retinal cultures, P2X receptor stimulation causes RGC death, suggesting possible involvement of NLRP3 inflammasome and caspase-1.[159]

RGC axon degeneration after central retinal artery occlusion is mediated by the mitochondrial intrinsic apoptotic pathway[160] – cytosolic Bax, a pro-apoptotic Bcl-2 family member, levels are decreased at 3 and 6 h post injury, whereas mitochondrial Bax levels are elevated at 3, 6 and 24 h, suggesting that Bax translocates to the mitochondria.[160] In addition, cytosolic Cytochrome *C* levels are elevated at 3 h post injury but not at 6 and 24 h, and cleaved caspase-9 levels are elevated at 3 h.[160]

RGC are protected by intravitreal caspase-6 and -8 inhibitors (z-VEID-fmk and z-IETD-fmk) and siRNA against caspase-6 and -8 (siCASP6 and siCASP8) after I/R injury.[161] Two different siRNA were used for each caspase making off-target effects unlikely. Caspase-6 inhibition may act indirectly by increasing retinal glial CNTF production.[96] Two weeks after ischaemia, z-VEID-fmk (caspase-6, but also -3 and -7) and z-IETD-fmk (caspase-8 but also -3, -6, and -10) protect only a small proportion of RGC, whereas both siCASP8 and siCASP6 administration elevate RGC survival by ~ 60%.[161] This suggests that small peptide inhibitors are less effective, as they act as a competitive inhibitor for the caspase substrates, whereas siRNA gene knockdown reduces caspase gene expression and could affect non-apoptotic caspase roles, such as caspase-8 in complex IIb, 'FADDosome', 'ripoptosome' and inflammasome formation.[20]

Glaucoma. Glaucoma is a complex, multifactorial disease affecting >60 million people worldwide[162] and is associated with raised IOP causing RGC death. Genetic background[163] and age[164] are also associated with disease development. Glaucoma is currently treated by IOP control; however, there is an unmet clinical need for a neuroprotective treatment.

Acute severe IOP elevation induces I/R injury, but models use less severe IOP elevation to simulate glaucoma, include the photocoagulation laser model,[165] injection of hypertonic saline solution,[166] injection of paramagnetic microspheres into the anterior chamber, suture-pulley compression,[167] intracameral transforming growth factor beta (TGF-β) injection[168] and AAV-TGF-β transfection to induce trabecular meshwork fibrosis.[169]

Apoptotic caspases -3, -8 and -9 are cleaved in RGC after a period of elevated IOP[166,167,170–176] and inflammatory caspases -1, -4 and -12 are also upregulated.[170]

In response to acute elevated IOP, NLRP3 inflammasome and IL-1β production are induced,[177,178] mediated through high-mobility group box-1 (HMGB1) via the NF-κB pathway.[178] HMGB1 promotes NLRP3 and ASC elevation leading to caspase-1 maturation. Caspase-8 acts upstream of the NF-κB HMGB1-caspase-8 pathway and induces the activation of NLRP3 and IL-1β production.[178] Toll-like receptor 4 (TLR4) activation increases macrophage caspase-8 expression upregulating IL-1β though the NF-κB pathway[178] and causes RGC death through the extrinsic pathway. Caspase-8 inhibition, using intravitreal z-IETD-fmk, reduces RGC death through NLRP1 and NLRP3 downregulation, though inhibition of a direct effect of caspase-8 (or other caspases) inhibition on the extrinsic apoptotic pathway is not excluded. Caspase-8 inhibition completely suppresses retinal IL-1β expression, but caspase-1 inhibition, using z-YVAD-fmk, does not, suggesting that caspase-8 regulates IL-1β expression through caspase-1-dependent and -independent pathways.[177]

Primary open-angle and normal-tension glaucoma patients display serum autoantibodies against retinal and ON antigens.[179–182] A 'glaucoma-like' syndrome, without direct damage to the retina or ON, has been induced using immunisation of ON homogenate causing RGC degeneration,[179,183] with increased GCL full-length caspase-3 expression at 14 and 22 days after immunisation.[179] However, RGC numbers did not decline until 22 days after immunisation.[179]

Diabetic retinopathy. RGC degenerate early in the disease process in the human diabetic retinopathy (DR) retinae demonstrated by scanning laser polimetry showing reduced RNFL thickness in DR patients compared with healthy controls.[184–186] TUNEL-positive RGC are increased in diabetic rats and in human postmortem retinae[187] and cleaved caspase-3, caspase-9, Fas and Bax localise to RGC.[188,189]

Diabetes mellitus develops in the Akita, insulin gene mutation (Ins2) mouse, after streptozotocin (STZ; toxic to β cells) administration, and in the Otsuka Long-Evans Tokushima fatty rats (OLETF; develop insulin resistance).[190–193] In STZ diabetic mice, retinal caspase activity (assessed with a variety of non-specific substrates) is increased 8 weeks after induction and GCL counts are reduced by 20–25% 14 weeks after induction, with TUNEL positivity and cleaved caspase-3 in the GCL, suggesting RGC apoptosis.[192,194] Caspase-2, -8 and -9 activity (using substrate sequences VDVAD, IETD and LEHD) transiently increases initially. By 4 months, caspase-3 activity increases and caspase-1, -3, -4 and -5 activities remain elevated,[194] corroborated by elevated cleaved caspase-8 and -3 levels in whole retinal lysates[195] and caspase-3 GCL immunolocalisation.[196] In primary retinal explants exposed to high glucose media, there are more cleaved caspase-3- and -9-positive RGC compared with explants in normal glucose media.[197]

CASPASES AND RGC AXON REGENERATION

In addition to promoting RGC survival, caspases promote RGC axon regeneration after ON injury. Pharmacological inhibition of caspase-6 and -8, using z-VEID-fmk and z-IETD-fmk, provide RGC neuroprotection and promote limited RGC axon regeneration,[61] with few axons extending >1000 µm beyond the lesion site. Similarly, few RGC axons regenerated through the lesion site with inhibition of caspase-6 by a dominant negative (CASP6 DN)[96]; however, combined suppression of caspase-2 and -6 using siCASP2 and CASP6 DN promoted significant regeneration, with an average of 195 ± 9 axons growing beyond 1000 µm.[96] Although caspase-6 is localised to RGC and some microglia, the neuroprotective and pro-regenerative effects of caspase-6 inhibition are mediated indirectly by CNTF upregulation in retinal glia and are blocked by suppression of gp130 and the JAK/STAT pathway.[96] These studies reveal a novel non-apoptotic role for caspases and warrants further investigation.

CONCLUSION

Postmitotic CNS neurons, including RGC, do not regenerate their axons after trauma or injury; hence RGC trauma or disease can lead to permanent visual loss. Understanding the signalling pathways in RGC injury is vital for the development of therapeutic interventions, such as pharmacological inhibitors, RNA interference technology or gene therapies. Caspases, a family of cysteine aspartate proteases, mediate RGC death in physiology, such as during development, as well as trauma and disease, and their inhibition can prevent RGC death. Caspase-3 is implicated during RGC developmental pruning, whereas most apoptotic and inflammatory caspases are implicated in trauma and disease, with siRNA knockdown of caspase-2 providing the greatest neuroprotection after axotomy. Non-apoptotic roles of caspases, such as inflammatory pyroptotic death or facilitating formation of necroptotic complexes are also critical in RGC death. Caspases also have a novel role in RGC axon regeneration; in particular, caspase-6 inhibition mediates regeneration indirectly through CNTF upregulation in retinal glia. Understanding the key pathways for caspase-dependant RGC death is fundamental to the development and effective translation of neuroprotective treatments from preclinical studies to clinical practice.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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